Daily Supplementation with 25 μg Cholecalciferol Does Not Increase Calcium Absorption or Skeletal Retention in Adolescent Girls with Low Serum 25-Hydroxyvitamin D1–4

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Abstract

In healthy adolescents, cross-sectional studies show either no or negative relationships between serum 25-hydroxyvitamin D [25(OH)D] and calcium (Ca) absorption. Using a 2-period metabolic balance study, the effect of vitamin D supplementation on retention in adolescent girls was investigated. Eleven girls aged 12–14 y with a mean entry serum 25(OH)D of 35.1 nmol/L consumed a controlled intake (providing 5 μg vitamin D and 1117 mg Ca/d) for two 3-wk metabolic balance periods separated by a 1-wk washout period. Sunlight exposure was minimized by sunscreen with a sun protection factor ≥ 15. After the first metabolic balance period, participants received 25 μg/d cholecalciferol supplementation for 4 wk. Fractional Ca absorption was measured in each metabolic balance period using a stable Ca isotope method. All urine and fecal samples were collected and analyzed to measure net Ca absorption and Ca retention. Paired t tests and correlations were used to analyze the data. Daily supplementation with 25 μg vitamin D resulted in a mean increase in serum 25(OH)D of 13.3 nmol/L (P < 0.01) but a decrease in fractional Ca absorption of 8.3% (P < 0.05) and no significant change in fasting serum 1,25-dihydroxyvitamin D, parathyroid hormone, net Ca absorption, or Ca skeletal retention. In pubertal girls with vitamin D status considered insufficient in adults, vitamin D supplementation of 25 μg/d for 4 wk did not improve fractional Ca absorption, net Ca absorption, or Ca retention.


Introduction

Maximizing calcium (Ca) retention during adolescence to achieve optimal peak bone mass is an important strategy to prevent osteoporosis in later years. This can be achieved through raising net Ca absorption by increasing dietary Ca (1,2) or through raising Ca absorption efficiency by increasing 1,25-(OH)2D [1,25(OH)2D] (3,4). 1,25(OH)2D, the physiological ligand for the vitamin D receptor produced in the kidney under the control of parathyroid hormone (PTH), stimulates active Ca absorption in the gut (5). In the intestinal cells, 1,25(OH)2D binds to the vitamin D receptor and induces transcription of intestinal Ca transport proteins, including TRPV6, calbindin-D9k, and PMCA1b (6). In adults, oral vitamin D supplementation in women with serum 25-hydroxyvitamin D [25(OH)D] < 60 nmol/L increased fractional Ca absorption (7–9), but cross-sectional studies show no relationship between serum 25(OH)D and Ca absorption above 10 nmol/L (10,11). In cross-sectional studies in children, fractional Ca absorption, net Ca absorption, and Ca retention were not positively related to serum 25(OH)D across a wide range of serum 25(OH)D values (12–16). The objective of this study was to determine the effect of vitamin D supplementation on Ca absorption and Ca retention in adolescent girls. We tested the hypothesis that supplementation with 25 μg/d cholecalciferol, a standard supplement dose, for 4 wk would increase Ca absorption and retention in adolescent girls with baseline serum 25(OH)D below 50 nmol/L.

Methods

Study design. Girls were recruited to participate in two 21-d balance studies with a 7-d washout period in between the 2 studies at Purdue University (40.5°N) during the summer of 2007. Participants were
supplemented with 25 μg vitamin D for 4 wk (washout and second balance study). Due to the 3-wk half-life of 25(OH)D (17), a randomized order design was not possible. Because the Carcopolynylene glycol (PEG) ratio becomes constant after 6 d in adolescents (18), the first 7 d served as the equilibrium period before determination of Ca balance and absorption.

Participants. Thirteen healthy girls, aged 12–14 y, from a sample of 51 adolescents with the lowest vitamin D status were selected [mean serum 25(OH)D of total participants recruited: 48.9 nmol/L; mean of those selected: 35.1 nmol/L] for this vitamin D intervention study. Screening questionnaires were used to determine medical history, physical activity, and habitual diet and a 6-d food record was completed. Food records were analyzed for their Vitamin D (2-site immunoassay, University of Minnesota, Minneapolis, MN). Exclusion criteria included history of use of medications that influence Ca metabolism, smoking, fracture in the past 6 mo, and pregnancy. Girls completed a self-evaluated sexual maturation questionnaire to assess Tanner stage (19). The protocol was approved by the Institutional Review Board at Purdue University. Written consent and assent were obtained from the legal guardian and participant, respectively.

Diet. The targeted total Ca intake was 975 mg/d, 560 mg/d from diet and 325 mg/d from a supplement (as CaCO3), and the analyzed mean values of the diet and supplement were 812 mg/d and 299 mg/d, respectively, resulting in a mean total Ca intake of 1177 mg/d. A 4-d cycle menu providing 3 meals and 2 snacks daily was used to control for Na (2.3 g/d), P (1100 mg/d), protein (70 g/d), fat (73.6 g/d), fiber (10 g/d), and vitamin D (5 μg/d).

Participants were given supplements of 25 μg cholecalciferol per capsule and were instructed to take 1 capsule daily during the 4-wk intervention. Serum PTH changes in response to the oral Ca load were analyzed by calculating the 5-h area under the curve (AUC) by the trapezoidal rule. The PTH concentration at 0, 0.5, 1, 3, and 5 h corresponds to PTH1, PTH2, PTH3, PTH4, and PTH5, respectively. The targeted total Ca intake was 975 mg/d, 650 mg/d from diet and 305 mg/d from supplements (as CaCO3). Three-hour post-dose sera were digested and analyzed for isotope ratio by inductively coupled plasma mass spectrometry (22).

Biochemical marker analysis. Blood samples collected at the beginning and end of each balance period were analyzed for serum 25(OH)D (RIA, DiaSorin), 1,25(OH)2D (RIA, DiaSorin), PTH (2-site immunoassay, Nichols Institute Diagnostics, inter-assay CV 7.1%), bone-specific alkaline phosphatase (BAP) and serum osteocalcin (OC) (ELISA and immunoradiometric assay, respectively; Quidel; CV 4.1 and 6.7%, respectively). Cross-linked N-telopeptide (NTx) (OsteX Intl.; CV 7.5%) and free deoxypyridinoline (Quidel; CV 7.6%) in urine from fasting participants was analyzed by ELISA.

Sample collection and analysis. All urine and fecal samples were collected from d 4 to 21 of each balance study in 24-h pools. Diet, fecal, and urine samples were analyzed for Ca by inductively coupled plasma optical emission spectrometry (Optima 4300 DV, Perkin Elmer Instrument) as previously described (20).

Total collection compliance of urine was assessed by urine creatinine measured by enzymatic colorimetric assay (COBAS Integra, Roche Diagnostics). Fecal compliance was assessed by percent recovery of fecal PEG measured by turbidimetric assay (21).

Fractional Ca absorption test. Fractional Ca absorption was performed near the end of each balance study with a single isotope and 3-h serum samples. Participants were given a breakfast containing 10 mg of 44Ca and 3-h serum samples. Participants were given supplements of 25 μg cholecalciferol per capsule and were instructed to take 1 capsule daily during the 4-wk intervention. Serum PTH changes in response to the oral Ca load were analyzed by calculating the 5-h area under the curve (AUC) by the trapezoidal rule. The PTH concentration at 0, 0.5, 1, 3, and 5 h corresponds to PTH1, PTH2, PTH3, PTH4, and PTH5, respectively. Participants were given supplements of 25 μg cholecalciferol per capsule and were instructed to take 1 capsule daily during the 4-wk intervention. Serum PTH changes in response to the oral Ca load were analyzed by calculating the 5-h area under the curve (AUC) by the trapezoidal rule. The PTH concentration at 0, 0.5, 1, 3, and 5 h corresponds to PTH1, PTH2, PTH3, PTH4, and PTH5, respectively. The targeted total Ca intake was 975 mg/d, 650 mg/d from diet and 305 mg/d from supplements (as CaCO3). Three-hour post-dose sera were digested and analyzed for isotope ratio by inductively coupled plasma mass spectrometry (22).

Fractional Ca absorption was calculated by an equation for single oral isotope 3-h post-dose serum samples validated against a double isotope method in the same age group as studied here (23).

\[
\text{Fractional Ca absorption} = \frac{1.9458(X - 0.256) - 1.6004}{e^{-0.1919(TS)}},
\]

where \(X\) = 44Ca-specific activity [fractional dose/Ca (g)]; \(BSA = \text{body surface area} (m^2) = [0.202447 \times \text{weight (kg)}]^{0.425} \times \text{height (m)}^{0.725} \); \(TS = \text{breast Tanner stage} \). 44Ca-specific activity is the fraction of the 44Ca dose detected in serum divided by total serum Ca. This equation was not influenced by serum vitamin D or PTH (23).

PTH suppression test. Near the end of each balance study, a test of serum PTH suppression following a fixed oral load of Ca (299 mg of Ca as CaCO3) with breakfast was performed to indirectly measure Ca absorption. Blood samples were collected 0, 0.5, 1, 3, and 5 h after consumption of the Ca tablet through venous catheters. Serum PTH changes in response to the oral Ca load were analyzed by calculating the 5-h area under the curve (AUC) by the trapezoidal rule. The PTH concentration at 0, 0.5, 1, 3, and 5 h corresponds to PTH1, PTH2, PTH3, PTH4, and PTH5, respectively. The equation used to calculate PTH AUC:

\[
\frac{\sum_{i=2}^{n} (PTH_i + PTH_{i-1})}{2(T_i - T_{i-1})},
\]

where PTH is expressed in ng/L and \(T_i\) is expressed in h.

Ca balance. The last 14 d of each balance period served as the experimental period. Ca retention was calculated as: Ca retention = Ca intake – (fecal Ca + urine Ca). Net Ca absorption was calculated as: net Ca absorption = Ca intake – fecal Ca.

Sunscreen. To minimize cutaneous production of vitamin D by sunlight, sunscreen with sun protection factor ≈ 15 was applied throughout the study before outside activities and every 4 h when continuously exposed to the sun. Skin reflectance was measured at the beginning and end of the balance period with a spectrophotometer (CM-2600d, Minolta) on the middle of the forehead, midpoint of forearm, and upper inner arm in triplicate after cleansing with an alcohol swab. These sites are indicators of photo-exposed skin (mid forehead and mid forearm) and photo-protected skin (upper inner arm). Skin reflectance was measured in L*, a*, and b* levels, indicators of lightness, redness, and yellowness, respectively. Mean percent CV for L*, a*, and b* measurements was 1.05, 4.83, and 2.85, respectively. Individual typology angle (ITA), a measurement of skin pigmentation, was calculated using the following equation (24):

\[
\text{ITA} = 57.3 \times \tan^{-1}\left(\frac{L' - 50}{b'}\right),
\]

where the inverse tangent is expressed in degrees.

Statistical analysis. SAS v9.1 (SAS Institute) was used for all statistical analyses. Correlations were used to describe relationships between variables and paired t tests were used to compare measures made at different times. A missing serum 25(OH)D observation for 1 participant at the end of the first study period was replaced with baseline serum 25(OH)D from the same period and a missing baseline PTH for another participant in the second study period was replaced by the baseline value of the first study period. Summaries are reported as mean ± SEM unless indicated otherwise. Significance was set as \(P < 0.05\).

Results

Participants

A total of 13 girls completed the first study period. Only data from the 11 girls who completed both studies were used in the analysis. Baseline data for these participants are shown in Table 1.

Effect of vitamin D supplementation

Biochemistry. At the end of the first 3-wk balance period with no vitamin D, participants’ serum 25(OH)D reached 46.8 ± 4.1 nmol/L. Supplementation with vitamin D increased serum 25(OH)D by 13.3 nmol/L (\(P = 0.0012\) and reached 60.1 nmol/L (Table 2). There were no significant changes in serum 1,25(OH)2D, PTH, total Ca, phosphate, and OC (Table 2) or urine NTx:creatinine and free Dpd:creatinine. BAP decreased by 5.8 μg/L with vitamin D supplementation (\(P < 0.05\)).
**Fractional Ca absorption.** Fractional Ca absorption decreased \((P < 0.05)\) by 8.3% with vitamin D supplementation (Table 2; Fig. 1). There were no significant correlations between fractional Ca absorption and serum 25(OH)D, 1,25(OH)\(_2\)D, PTH, phosphate, BAP, or OC concentration with or without vitamin D supplementation. Changes between study periods in these serum metabolites were unrelated to the change in fractional Ca absorption.

**Suppression of PTH as an indirect measure of Ca absorption.** The before \((107.2 \text{ ng} \text{/L})\) and after \((95.0 \text{ ng} \text{/L})\) vitamin D supplementation 5-h PTH AUC in response to a Ca load did not differ \((n = 9; P = 0.31)\) even when corrected for baseline \((P = 0.24)\). A log transformation of the PTH values gave a similar result. The correlation between PTH suppression and fractional Ca absorption, whether analyzed by study period or after pooling all data \((n = 20)\); 9 participants had 2 observations each and 2 participants had 1 observation each; \(r = 0.16; P = 0.50)\), was not significant; the same was true for their changes between balance studies \((n = 9; r = -0.45; P = 0.23)\).

**Ca balance.** Due to the few fecal samples during the first balance period, balance data for 1 girl were eliminated. Mean overall fecal compliance was 80.6% as assessed by PEG recovery, with no difference between balance studies. This represents very good compliance, considering up to 15% of PEG may be absorbed \((18)\).

Net Ca absorption, Ca retention, and fecal Ca were unaffected by vitamin D supplementation (Table 2; Fig. 1). Urinary Ca excretion increased significantly during vitamin D supplementation whether expressed as the absolute amount or as the Ca:creatinine ratio. Net Ca absorption, Ca retention, urine Ca, and fecal Ca were not correlated with fractional Ca absorption for each balance study or changes between studies.

### Skin reflectance

Due to the small number of black \((n = 1)\) and multiracial \((n = 1)\) participants, the skin reflectance measures were analyzed only for whites and Asians combined. During the control balance period, forearm \((\text{indicator of genetic skin color and tanning})\) and upper arm \((\text{indicator of genetic skin color})\) ITA values decreased by 0.139 \((P = 0.03)\) and 0.266 \((P = 0.04)\), respectively, indicating that their skin became darker at these sites during this time period despite use of sun protection. During the vitamin D supplementation period, only upper arm ITA decreased by 0.197 \((P < 0.05)\). Forehead ITA, another indicator of genetic skin color and tanning, did not significantly change.

Serum 25(OH)D or its changes and skin reflectance measurement values \((L^*, a^*, b^*)\), or ITA) or changes were not correlated, regardless of measurement site at each corresponding time point. Fractional Ca absorption correlated with forehead ITA \((r = -0.79\) and \(-0.75\), for first and second balance periods,
Discussion

Daily supplementation of 25 μg cholecalciferol for ~4 wk to adolescent girls with low vitamin D status increased serum 25(OH)D and urine Ca excretion but did not increase fractional Ca absorption or alter other variables, including fasting serum 1,25(OH)2D, Ca and PTH, PTH suppression by a Ca load, net Ca absorption, and Ca retention.

The amount of increase in serum 25(OH)D following 25 μg/d supplementation of cholecalciferol in our participants is smaller than the 30 nmol/L predicted from the literature (25–28). Variation in the serum 25(OH)D response to vitamin D supplementation may also be due to the assay performance, particularly with the RIA (29).

The expected decrease in PTH was between 3 and 6 ng/L if serum 25(OH)D had increased the expected 30 nmol/L from large observational studies (16,30). The Ca provided to our participants (1100 mg Ca/d) may have been sufficient to suppress changes in PTH, because serum PTH is regulated by Ca intake. Serum PTH did not change in children supplemented with up to 10 μg/d vitamin D with a similar Ca intake (28) or in postmenopausal women with Ca intakes over 1200 mg/d supplemented with higher amounts of vitamin D (31–33). However, vitamin D supplementation did suppress the increase of serum PTH in male adolescents with a mean intake of 809 mg/d Ca from dairy products (26). Calcium intake in the present study was lower than the current recommendations based on white girls of 1300 mg Ca/d (34), but it was higher than the minimal intake for maximum calcium retention for Asian (Chinese American) adolescent girls, 970 mg Ca/d (20). The total Ca intake was slightly higher than the average Ca intake from the 2005–2006 NHANES data for girls in this age group [850 mg/d (35–37)] and our girls’ habitual intakes (593 mg/d).

The decrease in fractional Ca absorption with increased serum 25(OH)D is supported by cross-sectional studies in adolescents with low serum 25(OH)D (12,13,15). Also, in Nigerian rachitic children, fractional Ca absorption did not change with 3 d of vitamin D supplementation (38). Lee et al. (12) speculated that lower serum 25(OH)D may be a result of high conversion to 1,25(OH)2D, which increased vitamin D–mediated fractional Ca absorption. However, in our study, increasing serum 25(OH)D caused a decrease in fractional Ca absorption independent of fasting serum PTH or 1,25(OH)2D.

This intervention confirms our earlier observational report of no relation between serum 25(OH)D and net Ca absorption or Ca retention in adolescent girls (16). In that study, serum 1,25(OH)2D was significantly correlated with Ca absorption but did not explain any of the variability in Ca absorption once Ca intake was added to the model (16).

Several indicators of Ca absorption and utilization did not appear to agree. First, PTH suppression following a Ca load was not correlated with the stable Ca isotope method. The PTH suppression test can only distinguish between high Ca loads and placebo (39), whereas the isotope method directly measures Ca absorption over a wide range of doses. We used 299 mg for PTH suppression and 130 mg Ca for the isotope method to provide information over this range of load. This would have also had the effect of shifting the route of Ca absorption from primarily vitamin D–dependent active transport with the isotope method to more passive with the PTH suppression test. Similarly, vitamin D supplementation (100 μg/d) in adults with high Ca intakes (≥1200 mg/d) did not influence serum PTH suppression 2 h after an oral Ca load (40). Second, fractional Ca absorption and net Ca absorption from balance did not agree. Net Ca absorption underestimates true Ca absorption by endogenous secretion and is assessed on a Ca intake of 1100 mg/d for the whole day’s diet compared with the isotope method, which measured fractional Ca absorption from a 130-mg test load. The PTH suppression test and net Ca absorption from balance suggest that the decrease in active Ca transport as measured with the isotope method through increased vitamin D status does not improve Ca utilization when Ca intake is 1100 mg/d. Third, 24-h urine Ca excretion did not reflect fractional Ca absorption. Similarly, urine Ca did not reflect the change in fractional Ca absorption in adults (41). Urinary Ca comes from absorbed and resorbed Ca. An increase in urinary Ca in the absence of an increase in absorption suggests an increase in bone resorption. Serum 25(OH)D has been unrelated or inversely related to bone resorption markers in adolescents (16,25). Two bone resorption markers, the ratio of urinary NTx to creatinine (NTx:Cr) and Dpd to creatinine (Dpd:Cr), were unchanged in response to vitamin D supplementation. Further research is needed to resolve this effect of vitamin D on Ca and bone metabolism.

In our study, skin color was not a reliable tool to assess vitamin D status or its change, because change in skin pigmentation did not correlate with serum 25(OH)D changes during the first 3 wk of no vitamin D supplementation. This was also true in the larger cohort (total 51) studied simultaneously. In contrast, Armas et al. (42) reported a relationship between basic skin color (baseline L*) and change in serum 25(OH)D due to UV ray exposure in adults. Rockell et al. (43) observed that tanning (assessed by photo-exposed skin color adjusted for photo-protected skin color) was the strongest predictor of plasma 25(OH)D, regardless of race. The lack of correlation could have been due to the summer season in which we conducted the study or to the small sample size.

Limitations of our study include a small sample size, 1 increment of vitamin D supplementation, and 1 level of Ca intake. We powered our study on fractional Ca absorption using the single isotope method, which can detect a treatment effect of 0.1 with 80% power with 9 or more participants in a crossover design (23). Four weeks of vitamin D supplementation may not have been sufficiently long to detect maximal changes in Ca absorption and retention. Serum 25(OH)D status plateaus after 4 wk (44,45) to 6 wk (46–48) in adults. Cross-sectional studies in white, black, and Asian children do not show an effect of vitamin D status on Ca absorption (15,16); thus, it is unlikely that racial differences in Ca absorption (49) influenced the results of this study.

Our results indicate that daily supplementation of 25 μg vitamin D during a period where Ca intake is maintained at 1100 mg/d increases serum 25(OH)D and decreases fractional Ca absorption but does not influence the functional outcomes of net Ca absorption or retention in healthy adolescents with vitamin D status considered low in adults. Studies with a larger sample size using a range in vitamin D supplementation on Ca absorption and retention in adolescents are needed to address fully the efficacy of vitamin D supplementation on Ca metabolism.

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